Research Article

JOURNAL OF HEPATOLOGY

Hepatocyte exosomes mediate liver repair and regeneration via sphingosine-1-phosphate

Hiroyuki Nojima[1], Christopher M. Freeman[1], Rebecca M. Schuster[1], Lukasz Japtok[2], Burkhard Kleuser[2], Michael J. Edwards[1], Erich Gulbins[1,3], Alex B. Lentsch[1,*]

[1]*Department of Surgery, University of Cincinnati, College of Medicine, Cincinnati, OH 45267, USA;* [2]*Institute of Nutritional Science, University of Potsdam, Arthur-Scheunert Allee 114-116, 14558 Nuthetal, Germany;* [3]*Department of Molecular Biology, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany*

Background & Aims: Exosomes are small membrane vesicles involved in intercellular communication. Hepatocytes are known to release exosomes, but little is known about their biological function. We sought to determine if exosomes derived from hepatocytes contribute to liver repair and regeneration after injury.
Methods: Exosomes derived from primary murine hepatocytes were isolated and characterized biochemically and biophysically. Using cultures of primary hepatocytes, we tested whether hepatocyte exosomes induced proliferation of hepatocytes *in vitro*. Using models of ischemia/reperfusion injury and partial hepatectomy, we evaluated whether hepatocyte exosomes promote hepatocyte proliferation and liver regeneration *in vivo*.
Results: Hepatocyte exosomes, but not exosomes from other liver cell types, induce dose-dependent hepatocyte proliferation *in vitro* and *in vivo*. Mechanistically, hepatocyte exosomes directly fuse with target hepatocytes and transfer neutral ceramidase and sphingosine kinase 2 (SK2) causing increased synthesis of sphingosine-1-phosphate (S1P) within target hepatocytes. Ablation of exosomal SK prevents the proliferative effect of exosomes. After ischemia/reperfusion injury, the number of circulating exosomes with proliferative effects increases.
Conclusions: Our data shows that hepatocyte-derived exosomes deliver the synthetic machinery to form S1P in target hepatocytes resulting in cell proliferation and liver regeneration after ischemia/reperfusion injury or partial hepatectomy. These findings represent a potentially novel new contributing mechanism of liver regeneration and have important implications for new therapeutic approaches to acute and chronic liver disease.

Keywords: Liver injury; Sphingolipids; Sphingosine kinase; Ischemia/reperfusion; Transplantation.
Received 18 June 2015; received in revised form 17 July 2015; accepted 25 July 2015; available online 5 August 2015
* Corresponding author. Address: Department of Surgery, University of Cincinnati, College of Medicine, 231 Albert Sabin Way, ML 0558, Cincinnati, OH 45267-0558, USA. Tel.: +1 (513) 558 8674; fax: +1 (513) 558 8677.
E-mail address: alex.lentsch@uc.edu (A.B. Lentsch).
Abbreviations: SK, sphingosine kinase; S1P, sphingosine-1-phosphate; BrdU, 5-bromo-2-deoxyuridine; I/R, ischemia/reperfusion; ELISA, enzyme-linked immunosorbant assay; PCNA, proliferating cell nuclear antigen; H3-P, phosphorylated histone H3; FSBA, fluorosulfonylbenzoyladenosine.

Introduction

Liver regeneration is a compensatory process that replaces functional liver mass lost as a result of injury or disease. The mechanisms governing this process are highly complex and have been the focus of investigation for decades. The specific functions of numerous hormones, growth factors and cytokines have been identified over the temporal course of the regenerative process [1]. However, whether other forms of intercellular communication, such as exosomes, may participate in this process has not been investigated.

Exosomes are membrane nanovesicles (30–100 nm) released by cells into the extracellular environment upon fusion of multivesicular bodies with the plasma membrane [2–4]. Exosomes contain membrane components but also contain proteins, microRNAs and mRNAs [4–7]. A variety of cell types, including hepatocytes, secrete exosomes into body fluids such as blood and urine [5,8–10]. Previous studies have shown that exosome released from cells depends on the activity of neutral sphingomyelinase, releasing ceramide from sphingomyelin and thereby controlling the process of exosome formation and release [11,12]. An emerging interest in exosomes has focused on their potential roles as a form of intercellular communication via delivery of exosomal contents and modulation of cellular activities in recipient cells [5,6,13]. For example, B-lymphocytes and dendritic cells have been shown to release exosomes that can stimulate T cell proliferation and contribute to a robust immune response [14]. In contrast, tumor cells have been shown to release exosomes that promote tumor growth and metastasis [15], and hepatocytes infected with hepatitis C virus (HCV) have been shown to release exosomes that contain and transmit HCV to other hepatocytes [16–18]. As such, it appears that exosomes serve as a fundamental mechanism of cell communication for basic homeostasis that can be hijacked by malignant and virus-infected cells.

Here, we investigated the role of exosomes as a mode of intercellular communication in the processes of liver repair and regeneration. We demonstrate that exosomes produced by hepatocytes, but not other liver cell types, promote hepatocyte proliferation *in vitro* and induce liver regeneration *in vivo*. The mechanism of this effect was found to be due to sphingosine kinase 2 (SK2), which was contained within hepatocytes exosomes, but was absent in exosomes from other liver cell types.

Delivery of SK2 by exosomes induced proliferation in target hepatocytes via synthesis of intracellular sphingosine-1-phosphate (S1P). This represents a novel mechanism by which hepatocytes signal via exosomes in a paracrine fashion to promote liver repair and regeneration after injury.

Materials and methods

Mice

Male C57Bl/6 mice and SK2-knockout mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice used for experiments were 6–8 weeks of age. All animal use and procedures described were reviewed and approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the National Institutes of Health guidelines. All animals and resulting samples were assigned a number that did not reveal the group allocation so that analyses were performed by blinded investigators.

Liver cells and exosome isolation and characterization

Hepatocytes and Kupffer cells were isolated from mice as previously described [19], and 2×10^6 cells/5 ml Williams media supplemented with 5% exosome-free FBS (System Biosciences) were cultured in 50 mm plates. Cells were cultured overnight and then media was changed. Forty-eight hours later, media was harvested for exosome isolation. Primary liver sinusoidal endothelial cells from C57Bl/6 mice were purchased from Cell Biologics (Chicago, IL). 1×10^7 cells/10 ml endothelial cell medium (Cell Biologics) supplemented with 5% exosome-free FBS were cultured in T75 flasks. Media was changed when cells reached confluence and 48 h later media was harvested for exosome isolation. Exosomes were isolated using differential centrifugation and sucrose density-gradient methods as previously described [20,21].

Serum exosomes were isolated with Exoquick according to the manufacturer's protocol (System Biosciences). Briefly, blood was obtained by cardiac puncture and 125 µl of serum was collected and mixed with Exoquick. Samples were centrifuged at 1500 g for 30 min, followed by incubation overnight at 4 °C. The supernatant was decanted and the exosome pellet was resuspended in phosphate-buffered saline (PBS).

Exosome size was determined using a Zetasizer Nano (Malvern Instruments, Malvern, UK) and the number of exosomes was assessed by the CD81-antigen ELISA kit (System Biosciences). Exosome purity was assessed by electron microscopy and by Western blot of markers of early endosome (EEA-1), endoplasmic reticulum (Grp78), and exosome (Tsg101, CD81, and CD63) compartments, respectively.

Electron microscopy

Exosomes in PBS were fixed in 1.5 M sodium cacodylate buffer (pH 7.4) and were absorbed onto copper-mesh formvar grids (Electron microscopy Sciences, Hatfield, PA) and negatively stained by 2% uranyl acetate. Samples were observed using a H7650 transmission electron microscope (Hitachi, Tokyo, Japan) operated at an accelerating voltage of 80 kV. Images were taken with an AMT digital camera for data acquisition.

Hepatocyte proliferation

Hepatocyte proliferation *in vitro* was determined by DNA incorporation of 5-bromo-2-deoxyuridine (BrdU). Hepatocytes were treated with hepatocyte-derived exosomes for 24 h prior to assessing BrdU incorporation. Data were normalized by the amount of viable cells and expressed as a ratio compared with medium alone. A commercial BrdU cell proliferation ELISA system (Abcam, Cambridge, UK) was used for this assay.

For histologic analysis of hepatocyte proliferation, tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin prior to immunohistochemical staining for proliferating cell nuclear antigen (PCNA) or phosphorylated histone H3 (H3-P). Staining of PCNA was performed as previously described [22], and staining of H3-P was performed according to the manufacturer's instructions (Ser10; dilution of 1:200, Cell Signaling Technology, Danvers, MA). Sections were counterstained with hematoxylin and quantitation was performed based on the percentage of positive nuclei of 400–600 hepatocytes from 4–6 positive fields at high power (400×).

Hepatic ischemia/reperfusion (I/R) injury and partial hepatectomy

Mice were randomly assigned to undergo either sham surgery, I/R, or partial hepatectomy as previously described [23,24]. For I/R injury, sham mice underwent the same procedure without vascular occlusion. Mice were injected intravenously with exosomes or saline (vehicle control) 24 and 48 h after reperfusion. For partial hepatectomy, mice were injected intravenously with exosomes or saline (vehicle control) immediately after and 24 h after hepatectomy.

Exosome-hepatocyte fusion

Exosomes were labeled with 2 µM PKH67 (Sigma-Aldrich) for 5 min, washed and incubated for 24 h with cultured hepatocytes. The samples were washed and counterstained with DAPI, and analyzed by fluorescence microscopy.

Measurement of sphingolipid substrates and enzymes

Ceramide was quantified by kinase assays exactly as previously described [25].

Quantification of S1P in hepatocytes was determined by ELISA and mass spectrometry. An S1P ELISA (Echelon Biosciences) was performed according to the manufacturer's instructions. For mass spectrometry, S1P was extracted by a modified two-step lipid extraction. Briefly, cells were transferred into a glass tube and resuspended in 1 ml of medium. Then, 100 pmol C17-S1P as internal standard, 100 µl of a 3N NaOH solution, 1 ml of chloroform and 1 ml of methanol/HCl (99.8:0.2 v/v) were added. After separation, the aqueous phase was acidified with 100 µl concentrated HCl and extracted with 1.5 ml chloroform. The organic phase was evaporated and the dried lipids were resolved in 200 µl methanol. Sample analysis was performed by rapid resolution liquid chromatography/tandem mass spectrometry using a quadrupole time of flight 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive electrospray ionization mode. Chromatographic separations were performed by an X-Bridge column (C18, 4.6 × 150 mm, 3.5 µm particle size, 138 Å pore size, Waters GmbH, Eschborn, Germany). Elution was performed using a gradient consisting of eluent A (water/formic acid 100:0.1 v/v) and eluent B (acetonitril/tetrahydrofuran/formic acid 50:50:0.1 v/v/v). The precursor ions of S1P (m/z 380.2560) and C17-S1P (m/z 366.2404) were cleaved into the fragment ions of m/z 264.2700 and m/z 250.2529 respectively. Quantification was performed with Mass Hunter Software.

Neutral sphingomyelinase activity was measured by incubation of samples with 0.05 µCi per sample [^{14}C]sphingomyelin in 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM DTT, 0.2% Triton, 10 µg/ml each of aprotinin and leupeptin for 60 min at 37 °C. The [^{14}C]sphingomyelin was dried prior to analysis, resuspended in the assay buffer, sonicated for 10 min and an aliquot was added to the samples. The reactions were analyzed as above.

Neutral ceramidase activity was measured by incubating samples in 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2.5 mM DTT, 0.2% Triton, 10 µg/ml each of aprotinin and leupeptin and 0.1 µCi micellar [^{14}C16]-ceramide (ARC0831, 55 mCi/mmol). The substrate was dried prior to use, resuspended in the assay buffer and bath-sonicated for 10 min. Samples were extracted after 60 min in 200 µl H_2O and $CHCl_3$:CH_3OH:HCl (100:100:1, v/v/v). The lower phase was dried, samples were resuspended in $CHCl_3$:CH_3OH (1:1, v/v) and separated by thin-layer chromatography (TLC) using $CHCl_3$:CH_3OH:ammoniumhydroxide (90:20:0.5, v/v/v) as the developing solvent. The plates were analyzed using a Fuji-Imager and ceramidase activity was determined by conversion of radioactive ceramide into sphingosine and radioactive fatty acid.

To determine SK activity, samples were incubated with 500 pmol sphingosine in the presence of 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM $MgCl_2$, 1 mM ATP and 10 µCi [^{32}P]γATP for 60 min at 30 °C. Samples were extracted by addition of 20 µl 1N HCl, 800 µl $CHCl_3$/CH_3OH/1N HCl (100:200:1, v/v/v), 240 µl $CHCl_3$ and 2 M KCl. Phases were separated, the lower phase was collected, dried, dissolved in 20 µl of $CHCl_3$:CH_3OH (1:1, v/v) and separated on Silica G60 TLC plates using $CHCl_3$/CH_3OH/acetic acid/H_2O (90:90:15:5, v/v/v/v). The TLC plates were analyzed employing a phosphoimager. Sphingosine was quantified by comparison with a standard curve of C18-sphingosine and SK activity was calculated from the conversion of the standards to S1[^{32}P].